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Olpitrichum sphaerosporum: a new USA record and phylogenetic placement

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Abstract — *Olpitrichum sphaerosporum*, a dimorphic hyphomycete isolated from the foliage of *Juniperus chinensis,* constitutes the first report of this species in the United States. Phylogenetic analyses using large subunit rRNA (LSU) and internal transcribed spacer (ITS) sequence data support *O. sphaerosporum* within the *Ceratostomataceae*, *Melanosporales*.

Key words — asexual fungi, *Chlamydomyces*, *Harzia*, *Melanospora*

Introduction

Olpitrichum G.F. Atk. was erected by Atkinson (1894) and is typified by *Olpitrichum carpophilum* G.F. Atk. Five additional species have been described: *O. africanum* (Saccas) D.C. Li & T.Y. Zhang, *O. macrosporum* (Farl. Ex Sacc.) Sumst., *O. patulum* (Sacc. & Berl.) Hol.-Jech., *O. sphaerosporum,* and *O. tenellum* (Berk. & M.A. Curtis) Hol.-Jech. This genus is dimorphic, with a *Proteophiala* (*Aspergillus*-like) synanamorph.

Chlamydomyces Bainier and *Harzia* Costantin are dimorphic fungi also with a *Proteophiala* synanamorph (Gams et al. 2009). *Melanospora* anamorphs comprise a wide range of genera including *Acremonium*, *Chlamydomyces*,

Table 1. ITS and LSU sequences of *Olpitrichum sphaerosporum* and allied species used in this study

Taxon	Origin	Substrate	Voucher No.	GenBank No.		Reference
				ITS	LSU	
Olpitrichum sphaerosporum	USA	Foliage of *Juniperus chinensis*	UAMH 11865	KT221061*	KT221062*	This paper
Bertia tropicalis	Panama	Branch	SMH3513	—	AY695263	Huhndorf et al. (2004)
Chaetosphaerella phaeostroma	Costa Rica	—	SMH4257	—	AY695264	Huhndorf et al. (2004)
Corollospora maritima	—	—	PP1991	—	AF491260	Campbell et al. (2002)
Harzia acremonioides	USA	Seed	NRRL 54328	HQ288051	—	Wicklow DT; unpublished
	USA	Seed of *Zea mays*	NRRL 54327	HQ698593	—	Wicklow DT; unpublished
H. cameroonensis	Cameroon	Unknown creeper plant	CBS 136420 [T]	KF777163	KF777216	Crous et al. (2013)
Hirsutella stilbelliformis var. *myrmicarum*	UK	*Myrmica rubra*	IMI 396398 [T]	—	GQ866967	Evans et al. (2010)
Melanospora brevirostris	Canada	Alpine regosol	ATCC42427	—	AY015627	Zhang & Blackwell (2002)
M. singaporensis	Singapore	Soil	ATCC 38286 [T]	—	AY015629	Zhang & Blackwell (2002)
M. pascuensis	Easter I., Chile	Soil	IMI 378527 [T]	AJ011312	—	Stchigel et al. (1999)
M. tiffanyae	Canada	—	SMCD2222	FJ748921	FJ748915	Vujanovic V; unpublished
	USA	Contaminant	ATCC15515 [T]	—	AY015630	Zhang & Blackwell (2002)
M. zamiae	Indonesia	Air	ATCC 12340	—	AY046579	
Microascus trigonosporus	—	—	RSA 1942	—	U47835	Spatafora J; unpublished
Papulaspora sepedonioides	Canada	Fallen female cones of *Picea glauca*	UAMH 10707	—	EU518666	Davey et al. (2008)
Petriella setifera	Hong Kong	Wood panel in coastal water	CBS 437.75	—	DQ470969	Zhang et al. (2006)
Scortechinia conferta	USA	—	SMH2648	—	AY695272	Huhndorf et al. (2004)
Sphaerodes compressa	Nepal	Soil, cow dung	IMI212200	—	AY015633	Zhang & Blackwell (2002)
S. fimicola	USA	Sclerotium of *Aspergillus flavus*	NRRL 54804	JQ034510	—	Wicklow DT; unpublished
	Japan	Hare dung	ATCC26180	—	AY015628	Zhang & Blackwell (2002)
S. pseudofimicola	Australia	Forest soil	CBS 108937	JN709487		Li & Wang; unpublished
S. quadrangularis	Spain	Foil	CBS 112763 [T]	—	GQ354530	García et al. (2004)
Tolypocladium capitatum	—	*Elaphomyces* sp.	OSC71233	—	AY489721	Castlebury et al. (2004)
Vittatispora coorgii	India	Foil	BICC 7817 [T]	—	DQ017375	Chaudhary et al. (2006)
Xylaria hypoxylon [1]	USA	Wood	ATCC 42768	AY327477	U47841	Platas et al. (2004)

* new sequences; — information not available; [T] ex-type; [1] outgroup.

Harzia, *Papulaspora*, *Paecilomyces*, *Phialophora*, and *Proteophiala* (Ciferri 1958, Kendrick & DiCosmo 1979, Cannon & Hawksworth 1982, Rehner & Samuels 1995, Hawksworth et al. 1999, Davey et al. 2008, Kirk et al. 2008, Gams et al. 2009). Some *Papulaspora* species reportedly also have a *Proteophiala* synanamorph (Gams & Domsch 1969, Domsch et al. 1980, Seifert et al. 2011). The phylogenetic relationship among genera that have a *Proteophiala* synanamorphic state remains uncertain.

No teleomorph for *Olpitrichum* has yet been reported, and a search of GenBank showed no DNA sequence data for *Olpitrichum*. Here we present the LSU and ITS DNA sequences for *Olpitrichum sphaerosporum*. Among the genera sharing the *Proteophiala* synanamorph, we found no DNA sequence data for *Chlamydomyces*, two ITS sequences for *Harzia acremonioides*, one ITS and one LSU for *H. cameroonensis*, and one LSU for *Papulaspora sepedonioides*. The systematic assignment of *Olpitrichum* is ambiguous, except that the members of this genus belong to the asexual fungi of *Pezizomycotina*, *Ascomycota* (Index Fungorum 2015).

Several studies have been conducted to elucidate the phylogenetic relationships of *Melanospora* and its allies (Rehner & Samuels 1995, Zhang & Blackwell 2002, Zhang et al. 2006, Hibbett et al. 2007). These studies having indicated *Melanospora* as a sister taxon to the *Ceratostomataceae* (Zhang & Blackwell 2002, Hibbett et al. 2007), the new order *Melanosporales* was proposed in Hibbett et al. (2007) to accommodate *Melanospora* and its allies. Phylogenetic analysis using LSU sequence data indicated that *Papulaspora sepedonioides* Preuss also belongs to *Melanosporales* (Davey et al. 2008).

Olpitrichum sphaerosporum, isolated from the foliage of *Juniperus chinensis* in Windsor, Connecticut, constitutes the first record of this fungus in the United States. Here we shed new light on the placement of *O. sphaerosporum* based upon ITS and LSU DNA sequence analyses.

Materials & methods

A sample of *Juniperus chinensis* with a purported foliar disease was submitted to the Inquiry Office of The Connecticut Agricultural Experiment Station Valley Laboratory for diagnosis in February 2014. The sample was placed in a plastic bag with a piece of wet paper towel for three days at room temperature. Following incubation, a fungus grew on the necrotic part of the foliage. A sterile needle was used to transfer the fungal spores to a 2% malt extract agar (MEA) plate. Fungal isolates were grown on MEA at 25°C for 7 days. Conidiophores and conidia were mounted in 85% lactic acid. All microscopic observations were made under an Olympus BX40 microscope with Nomarski differential interference contrast optics. Photomicrographs were taken with an Olympus Microfire digital camera (Goleta, CA). Measurements of the fungal structures were statistically analyzed for means and standard deviations with 95% confidence interval of means.

DNA was extracted from a petri plate-grown colony according to the procedure in ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research, Irvine, CA, USA). Oligonucleotides ITS1 (5′–TCCGTAGGTGAACCTGCGG–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) were used with isolated genomic DNA to amplify a fragment corresponding to the small subunit rDNA internal transcribed spacer (ITS) region by polymerase chain reaction (PCR) (Gardes & Bruns 1993). Oligonucleotides 5.8SR (5′ TCGATGAAGAACGCAGCG 3′) and LR7 (5′–TACTACCACCAAGATCT–3′) were used to amplify the ITS region and a portion of the large subunit rDNA region by PCR (Gargas & DePriest 1996). The resulting PCR products were purified using QIA quick PCR Purification columns (Qiagen, Valencia, CA, USA). The ITS PCR product was sequenced using oligonucleotides ITS1, ITS4, ITS2 (5′–GCTGCGTTCTTCATCGATGC–3′), and ITS3 (5′–GCATCGATGAAGAACGCAGC–3′). The LSU PCR product was sequenced using primers LROR (5′–ACCCGCTGAACTTAAGC–3′), LR7 (5′–TACTACCACCAAGATCT–3′), LR5 (5′–TCCTGAGGGAAACTTCG–3′), LR3R (5′–GTCTTGAAACACGGACC–3′), and LR16 (5′–TTCCACCCAAACACTCG–3′). DNA sequence information was deposited to GenBank as accession KT221061 for the ITS region and KT221062 for the LSU region.

Sequence similarity searches and comparisons were conducted using MegaBLAST (Wheeler et al. 2003) against the NCBI nucleotide database. *Harzia*, *Olpitrichum,* and several additional allied genera were chosen for phylogenetic analysis (Table 1). *Xylaria hypoxylon* (ATCC 42768) was designated as outgroup.

Sequences were aligned using ClustalW (Thompson et al. 1994). Phylogenetic analyses were conducted using the neighbor joining and maximum likelihood procedures in MEGA6 (Tamura et al. 2013). A bootstrap test was carried out with 1000 replicates.

Results

Taxonomy

Olpitrichum sphaerosporum Matsush., Icon. Microfung. Matsush. Lect.: 103 (1975) Fig. 1

Colonies on MEA effuse, cottony, pale ochraceous to yellow-buff, attaining 100 mm in diam. at 25°C in 7 days; aerial hyphae abundant; some mycelia reaching the lid of the plate in 7 days. Conidiophores erect, simple or sparsely branched at right angles, (100–)150–400(–500) µm long, 4–6(–7) µm wide at the base and the middle, 4–6 septate, thick-walled, smooth. Terminal fertile part of conidiophores 5–15 µm long, often extending, with 3-6 denticle conidiogenous cells. Conidiogenous loci denticles arranged in irregular verticils, tapering to the apex, 4–15 µm long, 7–9 µm wide at the base, rarely constricted at the base, often branched and developing new denticles or extending and forming new conidiophores. Conidia unicellular, solitary, subhyaline to pale ochraceous, spherical or subspherical, smooth or verruculose, sometimes with a small papilla at the base, (18–)19–23(–25) × (15–)18–22(–25) µm (n = 30), with wall 0.5–1 µm thick. Conidial secession rhexolytic.

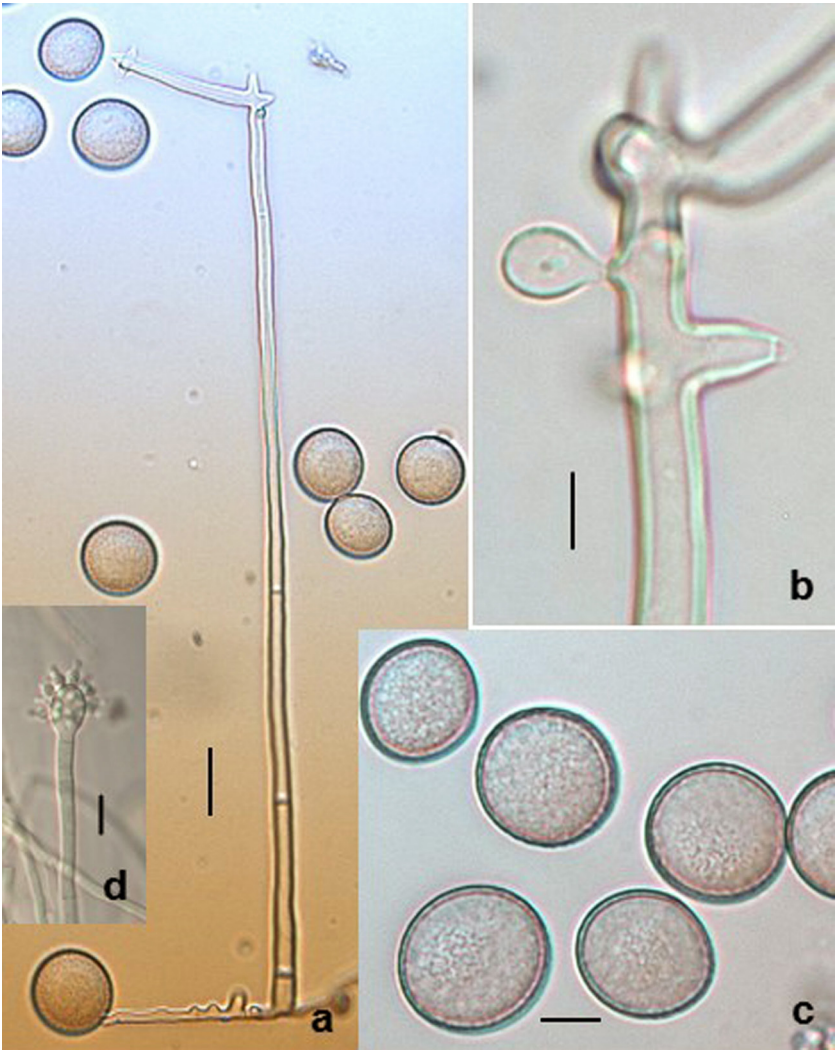

Fig. 1. *Olpitrichum sphaerosporum* (UAMH 11865) a. Conidiophore and conidia; b. Conidiogenous cells and an immature conidium; c. Conidia; d. *Proteophiala* synanamorph. Scale bars: a = 20 µm; b = 5 µm; c, d = 10 µm.

Synanamorph *Proteophiala* (*Aspergillus*-like), scattered in basal hyphae; conidiophores colorless, smooth, phialides 6–7.5 × 3–4 µm, conidia catenate, obovoid with a truncate base, colorless, smooth, 2–4 × 1.5–3 µm in diam.

Material examined: **USA, Connecticut**, Windsor, 41°51′04″N 72°39′39″W, dead foliage of *Juniperus chinensis* L. (*Cupressaceae*), Feb 24, 2014, coll. De-Wei Li, UAMH 11865 (DWL2014-6).

Hosts/substrates: saprobe, on dead foliage of *Juniperus chinensis*, dead pericarp of *Camellia japonica* L., decaying leaf of palm.

Distribution: Japan (Matsushima 1975), Mexico (Heredia Abarca & Arias Mota 2008), USA.

Note: *Olpitrichum sphaerosporum* represents a new record for the USA.

Phylogenetic analyses

Maximum likelihood and neighbor joining analyses produced the same results regarding the relationships within the *Melanosporales*. LSU sequence analyses indicated that *Olpitrichum sphaerosporum* and members of the *Ceratostomataceae, Melanosporales—Harzia*, *Melanospora*, *Papulaspora*, *Sphaerodes*, *Vittatispora*—form a monophyletic lineage (Fig. 2), clearly

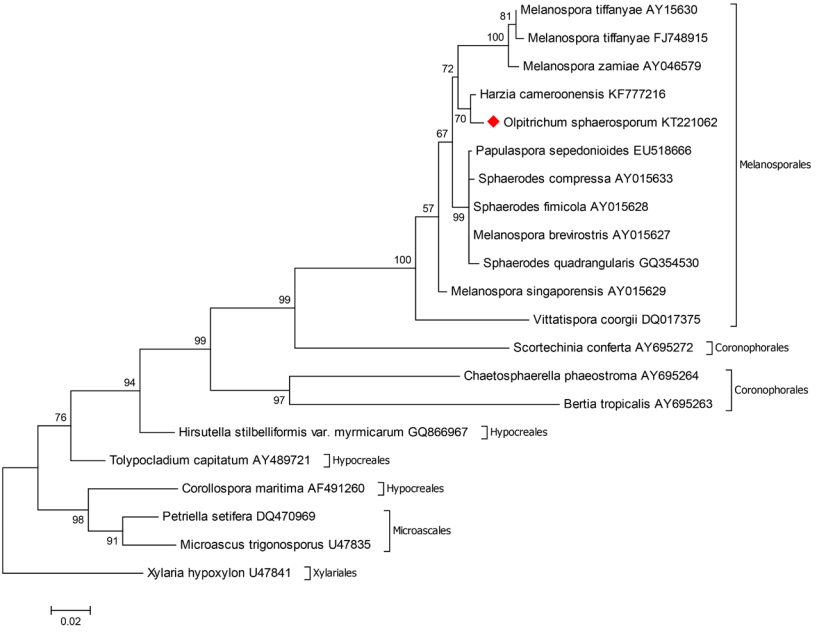

Fig. 2. LSU sequence maximum likelihood analysis of *Olpitrichum sphaerosporum* and related species (1000 bootstrap replicates; bs values indicated). Species names precede sequence accession numbers. *Xylaria hypoxylon* U47841 represents the outgroup. The scale bar indicates the number of expected changes per site.

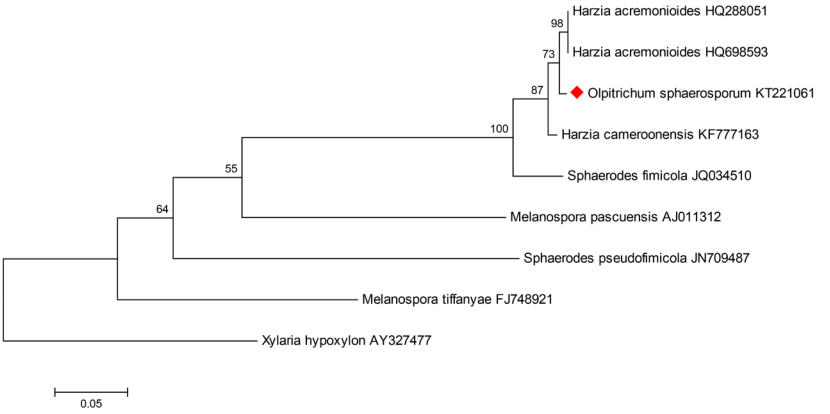

Fig. 3. ITS sequence maximum likelihood analysis of *Olpitrichum sphaerosporum* and allied fungi (1000 bootstrap replicates; bs values indicated). Species names precede sequence accession numbers. *Xylaria hypoxylon* AY327477 represents the outgroup. The scale bar indicates the number of expected changes per site.

supporting *Olpitrichum sphaerosporum* within the *Ceratostomataceae*. ITS sequence analyses resolved the phylogenetic relationship between *Olpitrichum sphaerosporum* and *Harzia* species more finely and grouped them in the same clade, indicating that *O. sphaerosporum* and *Harzia* (Fig. 3) are congeneric.

Discussion

This is the first report of *Olpitrichum sphaerosporum* from the United States. Among the six *Olpitrichum* species, only *O. sphaerosporum* develops spherical conidia. The Connecticut isolate differs slightly from the Japanese type isolate (Matsushima 1975) in having longer conidiophores.

The Connecticut isolate grows very slowly on DG18 media (less than 2 mm at 25°C in 7 days). It completely lost viability when stored in water at 4°C for 6 months, and only a few conidia survived when cryopreserved at –70°C for a year. This poses a challenge for culture maintenance.

Olpitrichum is morphologically similar to *Chlamydomyces* and *Harzia* (Holubová-Jechová 1974, Seifert et al. 2011) and all display a *Proteophiala* synanamorph (Seifert et al. 2011). *Chlamydomyces* and *Harzia* are both connected to the same sexual genus, *Melanospora* (Rehner & Samuels 1995).

As noted above, no rDNA sequence information for *Olpitrichum* and *Chlamydomyces* was available prior to this report. Both our phylogenetic

analyses (Figs 2, 3) and the morphologies of the asexual and sexual states suggest that phylogenetically *Olpitrichum*, *Harzia*, *Melanospora*, and *Sphaerodes* are closely related.

Harzia Costantin was erected by Costantin (1888) and typified by *H. acremonioides* (Harz) Costantin. *Harzia acremonioides*, *H. cameroonensis*, and *Olpitrichum sphaerosporum* were included in the phylogenetic ITS sequence analysis. As our results reveal that *Olpitrichum* and *Harzia* are closely related (Fig. 3), we conclude that either *Harzia* should remain a paraphyletic genus or that *Olpitrichum sphaerosporum* should be transferred to *Harzia.* With no culture of the type species, *Olpitrichum carpophilum,* currently available, it is premature to merge the two genera until the type specimen or verifiable material from the type species is available for DNA sequencing.

Previous studies have placed *Harzia*, *Melanospora*, *Sphaerodes*, *Vittatispora*, and *Papulaspora sepedonioides* within *Ceratostomataceae*, *Melanosporales* (Zhang & Blackwell 2002, Chaudhary et al. 2006, Davey et al. 2008, Lackner et al. 2014, Index Fungorum 2015), and Seifert et al. (2011) speculated that *Olpitrichum* might belong to *Ceratostomataceae*, *Melanosporales* according to its morphology. Our LSU sequence analysis groups *Olpitrichum sphaerosporum* in the same clade as the aforementioned genera (Fig. 2) and clear molecular evidence supports *Olpitrichum sphaerosporum* within the *Ceratostomataceae*, *Melanosporales.*

The studies of Zhang & Blackwell (2002) and Chaudhary et al. (2006) grouped *Sphaerodes* and *Melanospora* in the same clade, and Chaudhary et al. (2006) suggested that *Sphaerodes* and *Melanospora* are congeneric based on their phylogenetic analysis. Neither study included anamorphic genera, such as *Olpitrichum* and *Harzia*. Since the anamorph of *Sphaerodes* remains unknown and no DNA sequence data are available for the type species, *Sphaerodes episphaerium* (W. Phillips & Plowr.) Clem., the true phylogenetic relationships of *Sphaerodes* with *Melanospora* and other closely related asexual genera (*Olpitrichum* and *Harzia*) remain to be determined.

Olpitrichum, *Chlamydomyces*, and *Harzia* share the same *Proteophiala* synanamorph. However, the phylogenetic relationship of *Chlamydomyces* with *Olpitrichum* and *Harzia* remains unknown. From our results we could not determine the relationships among *Melanospora*, *Sphaerodes*, and *Papulaspora*. *Papulaspora* is a phylogenetically heterogeneous genus, and its members have been linked to numerous teleomorphs including *Ascobolus*, *Chaetomium*, *Cubonia*, *Hypomyces*, *Microthecium*, *Lophium*, *Melanospora*, *Neurospora*, and *Sordaria* (Seifert et al. 2011). *Papulaspora sepedonioides* is the type species of the genus. Thus, *Papulaspora* taxa not phylogenetically related to *Melanospora* should be excluded from *Papulaspora* and moved to other taxonomic groups.

However, as *Melanospora* is apparently also polyphyletic, further studies on these genera are necessary.

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